MUTANT *LACTOBACILLUS BULGARICUS* STRAINS FREE FROM BETA-GALACTOSIDASE ACTIVITY

These strains and ferments can be used for obtaining fermented dairy products from milk supplemented with glucose.

The present invention relates to novel variants of *bulgaricus* and to their use for preparing fermented dairy products.

Yogurts are conventionally obtained by fermentation of milk with a combination of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. During the fermentation, which is carried out at a temperature of approximately 40 to 45°C, these bacteria use mainly lactose as an energetic substrate, and produce lactic acid which causes the milk to coagulate; when the pH reaches a value of approximately 4.8 to 4.5, this fermentation step (also named "acidification") is terminated by cooling the product. This product is then kept in the cold during the remainder of the manufacturing and packaging process, and until its consumption.

However, the cooling does not completely stop the lactic acid fermentation; even when the product is kept at 4°C, a gradual increase in its acidity is observed over time.

This phenomenon, known as postacidification, is responsible for degradation of the organoleptic qualities of the product during its conservation.

The postacidification results essentially from the use by the bacteria, and mainly by *L. bulgaricus*, of the lactose remaining in the product at the end of the controlled acidification step. In order to avoid it, it has been proposed to use strains of *L. bulgaricus* which ferment lactose hardly or not at all.

One of the enzymes which are essential for the fermentation of lactose is β-galactosidase, which hydrolyzes lactose into glucose and galactose. It has

therefore been proposed, in order to obtain non-postacidifying strains of *L. bulgaricus*, to produce artificial mutants, or to select natural mutants, in which the activity of this enzyme is affected.

For example, patent EP 402 450 in the name of GENENCOR describes the production, by localized mutagenesis of the β-galactosidase gene, of conditional mutants of *L. bulgaricus*, in which the β-galactosidase, which is active during the fermentation at 40°C, loses its activity at the temperature or at the pH corresponding to the conditions of conservation of fermented dairy products.

Application JP 90053437 describes the production of an artificial mutant of *L. bulgaricus* which has completely lost the capacity to ferment lactose, and the selection of a natural mutant with decreased lactose fermentation capacity; these mutants are however both capable of developing and acidifying normally in the presence of *S. thermophilus*, on condition that the medium is supplemented with glucose. The subcultures of these mutants conserve their acidification characteristics, in milk lacking glucose, after 10 subculturings.

Patent EP 0518 096, in the name of the SOCIETÉ DES PRODUITS NESTLÉ, proposes to use, for manufacturing yogurt, poorly postacidifying mutants of *Lactobacillus bulgaricus* which have been preselected on the criterion of the deletion of a fragment of the β-galactosidase gene. The screening and characterization of these mutants are facilitated due to the fact that the presence of this deletion can be easily verified on restriction profiles. In addition, the deletions are known to be irreversible mutations, which makes it possible to easily obtain stable mutant strains from the parent strain. Patent EP 0518 096 describes two types of weakly postacidifying mutants selected in this way. The first have a deletion which affects only the β-galactosidase gene; when they are combined with *S. thermophilus* and cultured on milk, they exhibit,

even without the addition of glucose, growth and acidification properties which are comparable to those of the wild-type strain from which they are derived. The second have a larger deletion, stretching over at least 1 kb downstream of the β-galactosidase gene; when they are combined with *S. thermophilus*, they grow more slowly and acidify much less than the wild-type strain from which they are derived; the addition of glucose to the culture medium has only a slight influence on their acidification and postacidification properties.

Natural mutants in which the β-galactosidase is inactive are much more difficult to select and to maintain as pure cultures in the case of point mutations than in the case of deletion mutants; this is explained by the lower probability of a point mutation producing an inactive protein, by the greater difficulty in localizing and characterizing the point mutations using restriction profiles, and by the very high reversion rate.

The applicant has now found other natural mutants of *L. bulgaricus*, which do not carry a deletion in the gene encoding β-galactosidase, and which have advantageous technological characteristics. In the context of the present invention, a non-sense mutant, which is incapable of assimilating lactose, has been isolated from a culture of a wild-type *L. bulgaricus*. When combined with *S. thermophilus*, in culture on milk, it grows and acidifies much more slowly than the wild-type strain from which it is derived. Conversely, its growth and its acidification are virtually normal when the milk is supplemented with glucose.

A subject of the present invention is a mutant strain of *L. bulgaricus* lacking β-galactosidase activity, characterized in that it carries a mutation which introduces a non-sense codon into one of the coding sequences of the lactose operon, and in particular the sequence encoding β-galactosidase.

A strain of *L. bulgaricus* in accordance with the invention was deposited according to the Treaty of

Budapest, on January 14, 1998, with the CNCM (Collection Nationale de Cultures de Microorganisms [National Collection of Microorganism Cultures]) held by the Pasteur Institute, 25 rue du Docteur Roux, in Paris, under the number I-1968.

This strain has the following morphological and biochemical characteristics:

- Morphology: Gram-positive microorganism, immobile, isolated or short-chain, asporogenic, pleomorphic, thin bacilli.
- Metabolism: homofermentative, catalase (-).
- Fermentation of sugars: D-glucose (+), D-fructose (+), D-mannose (+), esculine (+).

The inventors have sequenced the lactose operon in the I-1968 mutant. The corresponding sequence is represented in the appended sequence listing under the number SEQ ID No: 1. The sequences of the translation products (permease and β-galactosidase) are represented under the numbers SEQ ID No: 2 and SEQ ID No: 3, respectively.

The analysis of this sequence reveals two point mutations: one, in the permease gene (position 122 of the sequence SEQ ID No: 1), induces an amino acid change (Lys → Asn); the other, in the β-galactosidase gene (position 4519 of the sequence SEQ ID No: 1), introduces a stop codon. Although conserving its active sites (positions 464 and 531), the β-galactosidase produced by this mutant is inactive. The inventors have also noted that this mutation remains stable after several series of subculturing, on a culture medium containing glucose. On the other hand, on a culture medium without glucose, this non-sense mutation reverts very rapidly at a rate of approximately 10^{-6}.

The present invention also encompasses mutant strains which are incapable of assimilating lactose and which are derived from the I-1968 strain. Such strains can, for example, be obtained by inducing other mutations in the lactose operon of the I-1968 strain, by site-directed mutagenesis.

A subject of the present invention is also a lactic ferment, in particular a yogurt ferment, characterized in that it comprises at least one strain of *L. bulgaricus* in accordance with the invention as defined above, preferably combined with at least one strain of *S. thermophilus*.

For the production of a ferment in accordance with the invention, any strain of *S. thermophilus* which is suitable for manufacturing yogurt can be used; the choice of one or more strains of *S. thermophilus* can be made as a function of the additional characteristics that it is desired optionally to confer on the finished product.

By way of example of strains of *S. thermophilus* which can be used in combination with a strain of *L. bulgaricus* in accordance with the invention, mention may be made of the following strains, deposited with the CNCM (Collection Nationale de Cultures de Microorganismes [National Collection of Microorganism Cultures]) held by the Pasteur Institute, 25 rue du Docteur Roux, in Paris:

- the strain deposited on August 25, 1994, under the number I-1470, and the strain deposited on August 23, 1995, under the number I-1620; these two strains are described in the European Application published under the number 96/06924;

- the strains deposited on December 30, 1994, under the numbers I-1520 and I-1521; these 2 strains are described in PCT international application WO 96/20607;

- the strain deposited on October 24, 1995 under the number I-1630; the characteristics of this strain are described in PCT international application WO 96/01701.

These strains can be combined mutually or with one or more other industrial strains of *S. thermophilus*.

The strain(s) of *S. thermophilus* is (are) combined with the strain(s) of *L. bulgaricus* in

accordance with the invention, in the same way and in the same proportions as in conventional yogurt ferments; the population of *L. bulgaricus* bacteria in accordance with the invention may, for example, represent between 10 and 90%, preferably between 20 and 50%, of the total bacterial population.

A subject of the present invention is also a method for preparing a fermented dairy product, characterized in that it comprises a step during which milk is fermented using a ferment comprising at least one strain of *L. bulgaricus* in accordance with the invention, in the presence of at least one sugar which can be assimilated by said strain; it can be in particular fructose, mannose and, preferably, glucose. Advantageously, said fermented dairy product is a yogurt.

The method in accordance with the invention is similar to conventional methods for preparing yogurt with regard to the main methods of implementation of the controlled acidification step; in particular, this acidification is carried out at a temperature of between 20 and 45°C, and preferably between 30 and 45°C, and "batchwise", i.e. in a single step and using a single fermentation tank.

The duration of this controlled acidification step is generally about 6 to 24 hours, and preferably about 6 to 16 hours; it is therefore longer than in the case of conventional methods for preparing yogurt (in which it is 3 to 5 hours at 44°C). Specifically, the strains of *L. bulgaricus* in accordance with the invention, even combined with *S. thermophilus*, grow and acidify much more slowly than the wild-type strains.

In addition, the rate of growth and acidification of the strains of *L. bulgaricus* in accordance with the invention varies very significantly depending on the amount of glucose added to the milk. This property makes it possible to control their growth and their acidification, by simply adding the desired amount of glucose at the start of fermentation.

The inventors have also observed that, when strains of *L. bulgaricus* or ferments in accordance with the invention are used, the acidification slows down considerably when the pH reaches the range of 4.8 to 4.5 (which corresponds to the pH range at which acidification is stopped in the case of a conventional method), and stabilizes, even if the milk is maintained at fermentation temperature, at a minimum pH. The value of this minimum pH depends essentially on the amount of glucose added.

This property makes it possible to reduce, or even to eliminate, the cooling phase used in conventional methods for manufacturing yogurt to stop the fermentation. It also eliminates the necessity of measuring the pH to determine the optimum moment for stopping the fermentation; for a given ferment and amount of added glucose, it is possible, without risk of overacidification, to stop the fermentation at the end of a given period, calculated as a function of the time required to reach the minimum pH. This makes it possible to have better control of the regularity of the final pH and of the texture for the product at the end of fermentation.

Advantageously, for the implementation of the method in accordance with the invention, and depending on the degree of acidification that it is desired to reach, the amount of glucose added to the milk prior to the fermentation is between 0.5 and 10 g/l, preferably between 0.5 and 5 g/l.

The fermented product obtained in this way can be conserved for several hours at a temperature close to the fermentation temperature, without a drop in pH, thereby making it possible to eliminate the installations for intermediate cold storage, and to increase the capacity of the fermentation tanks.

The implementation of the method in accordance with the invention makes it possible to reduce the postacidification in the fermented products during their longer term conservation. The degree of post-

acidification can vary depending on the composition of the ferment and the amount of glucose used. However, the postacidification is always clearly lower than that observed in the case of yogurts obtained with conventional ferments and methods.

For example, experiments carried out by the inventors have shown that, under the same conservation conditions (28 days of conservation at 10°C), the ΔpH (difference between the pH at D0 and the pH at D28) is between 0.05 and 0.4 in the case of the products obtained using a ferment in accordance with the invention, whereas it is always greater than 0.7 in the case of control ferments in which the strain of *L. bulgaricus* in accordance with the invention is replaced with a wild-type strain.

This weak postacidification is accompanied by good survival of the strains of the ferment; the population of *L. bulgaricus*, at the end of conservation, in the fermented product obtained in accordance with the invention is only slightly smaller than that of the control product.

A subject of the present invention is also the fermented dairy products which can be obtained by implementing a method in accordance with the invention.

These products can be conserved for a longer time and at higher temperatures than the products obtained using conventional methods, and have organoleptic properties which remain stable during conservation.

EXAMPLE 1: BIOCHEMICAL ASSAYING OF THE BETA-GALACTOSIDASE ACTIVITY OF A MUTANT IN ACCORDANCE WITH THE INVENTION

The β-galactosidase activity of the I-1968 strain was compared with that of the wild-type strain of *L. bulgaricus* (hereafter termed LbS) from which it is derived.

The bacteria are cultured overnight on MRS agar medium (MERCK) at 37°C, in an anaerobiosis jar (MERCK)

in the presence of an oxygen fixer (AnaerocultA, MERCK).

A 10-microliter loop (NUNC) of bacteria is resuspended in 1 milliliter of sterile water. The bacteria are lyzed with 2 cycles of vigorous shaking, 20 seconds at 5000 rotations per minute in the presence of glass microbeads (0.5 mm in diameter, BIOSPEC PRODUCTS), and then addition of 0.15 ml of chloroform. The mixture is shaken for 30 minutes at 37°C, and the volume is made to 2 ml with sterile water at 4°C. The beta-galactosidase activity is then measured: starting with 0.2 ml of the cell suspension, 1.2 ml of 0.067M NaH_2PO_4 buffer, pH 6.8; 0.05 ml of L-cysteine (SIGMA) at t0 0.05 ml of O-nitrophenyl-beta-D-galactopyranoside (SIGMA) are added. The enzymatic reaction is stopped after 0, 2, 5 or 10 min, with 1 ml of 10% Na_2CO_3 buffer, and, after centrifugation of the reaction medium, a measurement of the OD at 400 nanometers is performed on the supernatant.

The galactosidase activities of the LbS parent strain and of the I-1968 mutant in accordance with the invention, measured as a function of time, are given in Figure 1.

These results show that the β-galactosidase is totally inactive in the mutant in accordance with the invention.

EXAMPLE 2: STABILITY OF THE I-1968 MUTANT OF *L. BULGARICUS*

The stability of the I-1968 mutant was tested in media containing, as carbon sources, either a mixture of glucose and of lactose, or lactose only.

An I-1968 culture obtained on MRS medium containing glucose is subcultured on sterilized milk which is supplemented with yeast autolyzate (2 g/l) and which may or may not be supplemented with glucose (20 g/l). When a pH of 5.2 (coagulation of the milk) is reached, samples of each subculturing are taken, on which the capacity of the bacteria to ferment sugars, as well as the presence of β-galactosidase activity

(X-gal plate assay: white colonies = β-galactosidase minus; blue colonies = β-galactosidase plus), and analyzed.

The results are given in Table 1 below.

TABLE I

Medium	Milk + glucose (20 g/l)	Milk
Time to reach pH 5.2	6h00	20h00
Fermentation of sugars	glucose, fructose, mannose	lactose, glucose, fructose, mannose
X-gal plate assay	100% white colonies	20% white colonies 80% blue colonies

These results show that, in the presence of glucose, the I-1968 strain does not revert toward a strain capable of using lactose. Conversely, in a medium containing lactose as the only carbon source, rapid reversion of the I-1968 strain toward the original state is observed.

EXAMPLE 3: ACIDIFICATION, POSTACIDIFICATION AND SURVIVAL PROPERTIES OF THE I-1968 VARIANT OF *L. BULGARICUS* IN SYMBIOSIS WITH S. *THERMOPHILUS*: THE CASE OF A METHOD FOR MANUFACTURING A SET YOGURT (FERMENTATION IN A VENTILATED OVEN)

Yogurt ferments are prepared combining the I-1968 strain in accordance with the invention with various industrial strains of *S. thermophilus* (the strains of *S. thermophilus* used are hereafter termed ST1, ST2 and ST3).

By way of comparison, the ferments are prepared combining the LbS parent strain and the same strains of *S. thermophilus*.

For preparing the ferments, the strains are seeded separately and at 1% on the following composition:

Composition for 1 liter:

135 g of skimmed milk powder
2 g of yeast autolyzate
920 ml of distilled water
20 g of glucose (for the I-1968 strain only)
Hydration: 10 min

Pasteurization: 30 min at 95°C

The milk is then cooled to 44°C and inoculated, and then incubated at 44°C until an acidity of 85°D (degrees Dornic) for the streptococci and of 80°D for the lactobacilli is obtained.

The cultures are then cooled so as to obtain a ferment consisting of 80% *Streptococcus thermophilus* and of 20% *Lactobacillus bulgaricus*.

The ferments thus obtained are used to inoculate the following preparation:

Composition for 1 liter:

99% of milk

0, 1, or 2 g/l of glucose

Hydration: 10 min

Pasteurization: 10 min at 95°C

The milk is then cooled to 44°C and inoculated at 1%.

For each experiment, the composition of the ferment and the amount of glucose added are given in Table II below:

TABLE II

Experiment	Glucose g/l	Strains	Percentage
1	0	ST 3 ST 2 LbS	64% 16% 20%
2	0	ST 3 ST 2 I-1968	64% 16% 20%
3	1	ST 3 ST 2 I-1968	64% 16% 20%
4	0	ST 1 LbS	80% 20%
5	0	ST 1 I-1968	80% 20%
6	2	ST 1 I-1968	80% 20%

After inoculation, the milk is distributed into round-bottomed flasks and incubated at a temperature of 44°C. The acidification profile is monitored during the incubation. The products are uncurdled at pH 4.6 by cooling in a cold unit (16 hours at 4°C).

The products are then subjected to a conservation test at 10°C. In this test, the pH and Dornic acidity are measured after 1, 14, 21 and 28 days of conservation.

The acidification results (time to reach a pH of 4.6 and pH value at 24 h) are given in Table III below:

TABLE III

Experiment	Time to reach pH 4.6 (min)	Time to reach pH 4.5 (min)	pH at 24 h
1	215	236	3.67
2	550	778	4.33
3	416	507	4.26
4	225	241	3.67
5	660	>1500	4.54
6	390	465	4.35

The results of the conservation test at 10°C (monitoring of the pH and of the Dornic acidity) and the survival test (*S. thermophilus* and *L. bulgaricus populations*) at 28 days are given in Table IV below:

TABLE IV

Experiment	Storage time (days)	pH	Dornic acidity	*Streptococcus thermophilus* cells/ml	*Lactobacillus bulgaricus* cells/ml
1	1	4.41	101	7.25E+08	3.35E+08
1	14	3.98	140	ND	ND
1	21	3.95	145	ND	ND
1	28	3.9	148	7.35E+08	3.30E+08
2	1	4.5	93	5.60E+08	2.90E+07
2	14	4.23	110	Nd	ND
2	21	4.18	112	ND	ND
2	28	4.19	114	5.65E+08	1.87E+07
3	1	4.49	96	6.90E+08	7.45E+07
3	14	4.14	115	ND	ND
3	21	4.15	117	ND	ND
3	28	4.15	120	8.65E+08	6.30E+07
4	1	4.39	105	6.30E+07	4.40E+08
4	14	3.91	145	ND	ND
4	21	3.9	151	ND	ND
4	28	3.85	157	4.70E+08	6.30E+08
5	1	4.6	85	9.05E+08	6.70E+07
5	14	4.58	80	ND	ND
5	21	4.53	80	ND	ND
5	28	4.61	79	9.40E+08	7.00E+07

Experiment	Storage time (days)	pH	Dornic acidity	*Streptococcus thermophilus* cells/ml	*Lactobacillus bulgaricus* cells/ml
6	1	4.51	89	1.05E+09	1.96E+08
6	14	4.38	90	ND	ND
6	21	4.39	96	ND	ND
6	28	4.42	90	1.62E+09	1.91E+08

ND = Not Determined

These results show that the yogurts produced using the symbioses combining the I-1968 strain with one or two strains of *S. thermophilus* show extremely reduced postacidification with respect to the same symbioses with the LbS parent strain, while at the same time conserving an abundant population at the end of fermentation and good survival for 28 days at 10°C.

Stopping the acidification and maintaining the pH at around 4.6 to 4.5 for at least 24 hours at 44°C makes it possible, in the context of manufacturing stirred yogurt, to reduce or even eliminate the phase of cooling in a tank, which is conventionally used.